

2006

Defining the mechanism and functional consequences of PAI-2-mediated uPA/uPAR endocytosis

David R. Croucher
University of Wollongong

Follow this and additional works at: <https://ro.uow.edu.au/theses>

University of Wollongong

Copyright Warning

You may print or download ONE copy of this document for the purpose of your own research or study. The University does not authorise you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site.

You are reminded of the following: This work is copyright. Apart from any use permitted under the Copyright Act 1968, no part of this work may be reproduced by any process, nor may any other exclusive right be exercised, without the permission of the author. Copyright owners are entitled to take legal action against persons who infringe their copyright. A reproduction of material that is protected by copyright may be a copyright infringement. A court may impose penalties and award damages in relation to offences and infringements relating to copyright material.

Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

Unless otherwise indicated, the views expressed in this thesis are those of the author and do not necessarily represent the views of the University of Wollongong.

Recommended Citation

Croucher, David R, Defining the mechanism and functional consequences of PAI-2-mediated uPA/uPAR endocytosis, PhD thesis, School of Biological Sciences, University of Wollongong, 2006.
<http://ro.uow.edu.au/theses/632>

NOTE

This online version of the thesis may have different page formatting and pagination from the paper copy held in the University of Wollongong Library.

UNIVERSITY OF WOLLONGONG

COPYRIGHT WARNING

You may print or download ONE copy of this document for the purpose of your own research or study. The University does not authorise you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site. You are reminded of the following:

Copyright owners are entitled to take legal action against persons who infringe their copyright. A reproduction of material that is protected by copyright may be a copyright infringement. A court may impose penalties and award damages in relation to offences and infringements relating to copyright material. Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.

Defining the mechanism and functional consequences of PAI-2- mediated uPA/uPAR endocytosis

A thesis submitted in partial fulfillment of the requirements for
the award of the degree

Doctor of Philosophy

from

University of Wollongong



by

David R Croucher

Bachelor of Biotechnology (Honours 1st Class)

School of Biological Sciences
University of Wollongong
2006

I, David R Croucher, declare that this thesis, submitted in partial fulfillment of the requirements for the award of Doctor of Philosophy, in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

David R Croucher
12th October 2006

List of Publications

Croucher D, Saunders DN, Ranson M. The Urokinase/PAI-2 Complex: A New High Affinity Ligand for the Lipoprotein Receptor-Related Protein. *Journal of Biological Chemistry* 281: 10206-10213 (2006)

Al Ejeh F, **Croucher D**, Ranson M. Kinetic analysis of plasminogen activator inhibitor type-2:urokinase complex formation and subsequent internalisation by carcinoma cell lines. *Experimental Cell Research* 297(1):259-271 (2004)

Stutchbury TK, Al-ejeh F, Stillfried G, **Croucher D**, Allen B, Irving D, Andrews J, Links M, Ranson M. Preclinical evaluation of PAI-2-DTTA-²¹³Bi (alpha-PAI-2) in an orthotopic murine xenogenic model of human breast carcinoma. *Manuscript in press - Molecular Cancer Therapeutics*

Samson A, Niego B, Daniel P, Weiss TW, **Croucher D**, Lawrence DA, Medcalf RL. Tissue-type Plasminogen Activator Requires a Co-Receptor to Enhance NMDA receptor function. *Submitted to Journal of Biological Chemistry*

Croucher D, Saunders DN, Stillfried GE, Ranson M. Structural Basis of Differential Signaling by PAI-1 and PAI-2 in Breast Cancer: Implications for Metastatic Potential *Submitted to Cancer Research*.

List of Conference Presentations

Conference Oral Presentations

Croucher D, Saunders D, Ranson M.

PAI-2 is internalised by receptor mediated endocytosis.

IX International Workshop on Molecular and Cellular Biology of Plasminogen Activation, Isle of Capri, Italy (2003).

Croucher D, Saunders D, Ranson M.

Characterising the receptor mediated endocytosis of PAI-2.

International Society for Fibrinolysis and Proteolysis, Melbourne, Victoria (2004).

Croucher D, Saunders D, Leung H, Ranson M.

Structural basis of the differential signaling by initiated by PAI-1 and PAI-2: Implications for metastatic potential.

18th International Congress on Fibrinolysis and Proteolysis, San Diego, US (2006).

Samson AL, Niego B, Daniel PB, Weiss TB, **Croucher D**, Lawrence DA, Medcalf RL.

Tissue-type plasminogen activator can promote NMDA-induced neuronal stimulation via LDL receptor and plasmin-dependent mechanisms.

18th International Congress on Fibrinolysis and Proteolysis, San Diego, US (2006).

Conference Poster Presentations

Croucher D, Al-Ejeh F, Ranson M. The binding kinetics and cellular internalisation of PAI-2 by prostate cancer cells: Validating its use for targeted cancer therapy.

15th Lorne Cancer Conference, Lorne, Victoria (2003)

Al Ejeh F, **Croucher D**, Ranson M. Binding and internalisation characteristics of plasminogen activator inhibitor type 2 (PAI-2) on human breast and prostate cancer cell lines. IX International Workshop on Molecular and Cellular Biology of Plasminogen Activation, Isle of Capri, Italy (2003)

Croucher D, Saunders D, Ranson M. The PAI-2/urokinase complex: A new ligand for the low density lipoprotein receptor-related protein X International Workshop on Molecular and Cellular Biology of Plasminogen Activation, Washington DC (2005)

Croucher D, Saunders D, Ranson M. Annexin II, a novel cell surface receptor and avenue of endocytosis for PAI-2. Serpins Conference, Cairns, Australia (2005)

Lobov S, **Croucher D**, and Ranson M. Assessment of known/potential binding sites in the PAI-2 CD-loop for interaction with annexin II and endocytosis receptors. XVIIIth International Congress on Fibrinolysis and Proteolysis, San Diego, USA, (2006)

List of Tables

1.1 Reported affinities of plasminogen activation system components for receptors of the low density lipoprotein receptor family.....	42
3.1 The kinetics parameters of uPA and uPA:PAI-2 binding to LRP, measured by surface plasmon resonance.....	99
4.1 The kinetics parameters of PAI-1, PAI-1 ^{R76E} , PAI-2, uPA and uPA:serpin complexes binding to VLDLr, measured by surface plasmon resonance.....	123

List of Figures

1.1 Schematic diagram of the pericellular proteolytic activity of the plasminogen activation system.....	5
1.2 Schematic diagram of the structural and functional domains of uPA.....	9
1.3 The structural and functional domains of cell surface uPAR	13
1.4 The generalised mechanism of serpin inhibition	18
1.5 The structure of PAI-1	21
1.6 The structure of PAI-2.	24
1.7 Structures of the three endocytosis receptors responsible for the internalisation of uPA:PAI-1 complexes.....	35
1.8 A schematic diagram showing the main pathways of endocytosis.....	40
1.9 A brief schematic diagram of the interactions between uPAR, integrins, vitronectin, uPA, PAI-1, members of the LDLR family and the functional consequences of these interactions.....	46
2.1 The method of fluorescence quenching used to measure the internalisation of PAI-2	57
2.2 Cell surface expression profile of the PC-3 cell line.....	60
2.3 PAI-2 forms complexes with uPA at the surface of PC-3	62
2.4 The intracellular localisation of internalised PAI-2	63
2.5 Optimisation of the PAI-2:Alexa ₄₈₈ fluorescence quenching internalisation assay..	65
2.6 Confocal fluorescence microscopy analysis of the internalisation of PAI-2 by PC-3 cells	67
2.7 Quantification of PAI-2:Alexa ₄₈₈ internalisation by PC-3 cells	68
2.8 Cytotoxicity of the endocytosis inhibitors chlorpromazine and nystatin towards PC-3 cells	70

2.9	The effects of chlorpromazine and nystatin on transferrin internalisation	72
2.10	PAI-2 endocytosis can be prevented by inhibitors of clathrin dependent and independent processes	73
2.11	PAI-2 is internalised in a uPA, uPAR and LDLR specific manner by HEK 293 cells	74
2.12	PAI-2 is internalised in a partially annexin II dependent manner on PC-3 cells	76
2.13	The interaction of bovine annexin II heterotetramer and PAI-2, measured by surface plasmon resonance.....	76
3.1	PAI-2 endocytosis is inhibited by anti-LRP antibodies	91
3.2	The RAP sensitive co-localisation of internalised PAI-2 and LRP.	92
3.3	Ligand dot blot analysis of the interaction between uPA:PAI-2 and LRP	94
3.4	The purification of uPA:PAI-2 complexes	95
3.5	The formation of relaxed PAI-2.....	95
3.6	Surface plasmon resonance analysis of the interaction between uPA:PAI-2 and LRP	97
3.7	The binding of uPA and uPA:PAI-2 to LRP using surface plasmon resonance.....	98
3.8	Inhibition of uPA by PAI-2 results in enhanced clearance of the uPA:PAI-2 complex	100
3.9	Ligation to uPAR reduces but does not prevent the binding of both uPA and uPA:PAI-2 to LRP	102
4.1	uPAR and VLDLr mediate the endocytosis of uPA:PAI-2 by MCF-7 cells	116
4.2	Surface plasmon resonance analysis of PAI-1 and PAI-2 binding to VLDLr	117
4.3	Surface plasmon resonance analysis of the interaction between uPA and uPA:serpin complexes with VLDLr.....	121

4.4 Comparison of PAI-1 and PAI-2 VLDLr binding interfaces.....	123
4.5 VLDLr mediated internalisation of uPA:serpin complexes by MCF-7 cells	125
4.6 uPA:PAI-2 does not induce nuclear/cytoplasmic tyrosine phosphorylation of cellular proteins.....	127
4.7 Differential ERK phosphorylation in MCF-7 cells upon uPA:PAI-1 and uPA:PAI-2 stimulation.....	128
4.8 The correlation of phosphorylated ERK, VLDLr affinity and RAP sensitive uPA internalisation.....	129
4.9 uPA:PAI-2 does not stimulate cell proliferation of MCF-7 cells	130
5.1 Signaling events mediated upon the endocytosis of uPA:PAI-1 and uPA:PAI-2 by VLDLr.....	142

List of Abbreviations

Absorbance	A
Amino Terminal Fragment	ATF
Basement Membrane	BM
Bovine Serum Albumin	BSA
Deoxyribonucleic Acid	DNA
Disabled-1	Dab-1
Epidermal Growth Factor	EGF
Epidermal Growth Factor Receptor	EGFR
Ethylenediaminetetraacetic Acid	EDTA
Extracellular Matrix	ECM
Extracellular Signal-Regulated Kinase	ERK
Fluorescein Isothiocyanate	FITC
Foetal Calf Serum	FCS
Glycophosphoinositol	GPI
Gram	g
Gravity	<i>g</i>
High Molecular Weight	HMW
Horse Radish Peroxidase	HRP
Hour	h
4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid	HEPES
Immunoglobulin G	IgG
Association Rate	k_a
Dissociation Rate	k_d
Dissociation Constant	K_D

Kilodalton	kDa
Lipopolysaccharide	LPS
Litre	L
Low Density Lipoprotein Receptor	LDLR
Low Density Lipoprotein Receptor-Related Protein	LRP
Low Molecular Weight	LMW
Matrix Metalloprotease	MMP
Metre	m
Micro	μ
Milli	m
Minute	min
Molar	M
Nano	n
N-hydroxysuccinimide	NHS
Para-formaldehyde	PFA
Phenylmethanesulphonyl fluoride	PMSF
Phosphate Buffered Saline	PBS
Plasminogen Activator Inhibitor	PAI
Poly-Acrylamide Gel Electrophoresis	PAGE
Propidium Iodide	PI
Reactive Centre Loop	RCL
Receptor Associated Protein	RAP
Retinoblastoma	Rb
Revolutions per Minute	rpm
Sodium Dodecyl Sulphate	SDS

Second	sec
Serine Protease Inhibitor	Serpin
Standard Error of the Mean	SEM
Surface Plasmon Resonance	SPR
Tissue Plasminogen Activator	tPA
Transforming Growth Factor- α	TGF- α
Tris Buffered Saline	TBS
Tumour Necrosis Factor- α	TNF- α
Urokinase Plasminogen Activator	uPA
Urokinase Plasminogen Activator Receptor	uPAR
Very Low density Lipoprotein Receptor	VLDLr
Volts	V

Acknowledgements

After three and a half years or so there are a lot of people to whom I owe a debt of gratitude, but thanks must go firstly to my supervisor Marie Ranson. Marie always had time to listen, help out, offer suggestions or read the many piles of paper I placed on her desk. Marie allowed me the freedom and the funding to follow the many tangents I came up with along the way, but never allowed me to stray too far from the big picture. Working in Marie's lab gave me many experiences and opportunities I may have otherwise not had. An appreciation of good food, wine, coffee, and also learning to order something other than chicken, seem just as important as all the finer aspects of research.

My co-supervisor Darren Saunders was an invaluable sounding board for most of the experiments and theories that arose during my research. Thanks must go to him for the sheer amount of time and effort he put in to assisting me in every aspect of this thesis. Darren also provided me with a link to the facilities at the Garvan Institute without which much of this work would have been simply impossible.

My co-supervisor Tamantha Stutchbury was a continual source of wisdom for all research, personal and confectionary related matters. An indispensable part of any PhD.

Thankyou to all the members of the Ranson lab over the last three and a half years. They were always there to help, to have a laugh with or simply to put up with my reluctance to face the fact that it was my turn to do the washing up. Many thanks go to Gillian Hicks, Fares Al-Ejeh, Kara Perrow, Martina Sanderson-Smith, Fiona McKay, Sergei Lobov, Adam Lowe, Helen Leung, Jodi Lee, Blake Cochran and Laurel Morrissey.

Many thanks to those who looked after me whilst I was overseas. Particularly Niels Behrendt from the Finsen Laboratory in Copenhagen who went to great efforts to make me feel welcome in his lab and also to show me the sites of Denmark. Also to Chris Madsen, Nicolai Sidenius, Orla Cunningham and Macarena Lahores of IFOM in Milan who happily spent a lot of time helping me in their lab and showing me the restaurants and bars of Milan. Not that the latter took much convincing!

Thanks must also go to Rob Medcalf, Dudley Strickland, Dan Lawrence, Dieter Blaas, Phil Hogg, Teresa Compton and Peter Lenting, all of whom generously provided me with proteins and reagents without which I could not have completed this research.

Finally to my parents, Tom and Jenny, who supported, fed and encouraged me, in and out of home, without question and without reserve. To you I owe the biggest debt of gratitude of all. Thank you.

Abstract

Plasminogen is converted to its active form plasmin by two major serine proteases; the urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA). De-regulated plasmin formation is associated with tumour growth and progression. Whilst tPA is primarily involved in blood clot dissolution, uPA, along with its cell surface receptor uPAR, are commonly over-expressed at the leading edge of a tumour and by the tumour-associated stroma, contributing to plasmin formation, cell proliferation and migration. Soluble and receptor bound uPA is efficiently inhibited by two members of the serine protease inhibitor (serpin) superfamily; the plasminogen activator inhibitors type 1 (PAI-1) and 2 (PAI-2) (Serpins E1 and B2 respectively).

The purpose of this thesis was; **(1)** to examine the fate of cell surface bound PAI-2, a largely un-explored aspect of the plasminogen activation system, with particular focus on the possibility of the internalisation of uPA bound PAI-2; **(2)** to characterise the interaction between PAI-2, uPA:PAI-2 and any putative receptors involved in the internalisation of these proteins; and **(3)** to determine the functional consequences of the process of PAI-2 internalisation, in terms of regulation of uPA/uPAR levels and cell signaling responses.

Confocal microscopy and a novel flow cytometry based internalisation assay were used to both visualise and measure the interaction of PAI-2 with human carcinoma cancer cell lines. This data provided definitive proof that uPA bound PAI-2 was internalised into the endosomes and lysosomes of these cells, mediated through an interaction with endocytosis receptors of the low density lipoprotein receptor (LDLR) family. This finding may lead to the development of a more effective PAI-2 cancer therapeutic utilising the intracellular delivery of cytotoxins to cancer cells.

Surface plasmon resonance and further applications of the flow cytometry based internalisation assay were used to investigate the interactions of uPA:PAI-2 with two receptors of the LDLR family. This led to the characterisation of the interaction between uPA:PAI-2 and the low density lipoprotein receptor-related protein (LRP) and the very low density lipoprotein receptor (VLDLr). The biochemical analysis of these interactions, in comparison to that of uPA:PAI-1, led to the discovery of a novel difference in the kinetics and affinities of the interactions between uPA:PAI-1, uPA:PAI-2 and these receptors. Differing positive electrostatic potentials and conservation of a putative LDLR binding motif within helix D of these two serpins, specifically surrounding a conserved arginine residue, were implicated in the higher affinity of uPA:PAI-1 for these receptors.

The consequences of this variation in receptor binding were revealed using MCF-7 breast cancer cells. As previously demonstrated, the binding of the high affinity helix D site in uPA:PAI-1 to VLDLr on MCF-7 cells resulted in the propagation of intracellular signaling events and cell proliferation. As uPA:PAI-2 does not contain this high affinity site, these cell signaling events were not induced upon uPA:PAI-2 binding to VLDLr, however the complex was still efficiently endocytosed.

The data presented in this thesis therefore proposes a novel mechanism behind the disparity in patient prognosis associated with tumour expression of PAI-1 and PAI-2. The negative prognostic impact of PAI-1 may be mediated through the mitogenic effects of its high affinity LDLR binding site, whereas the positive prognostic impact of PAI-2 stems from its ability to efficiently inhibit and clear cell surface uPA without inducing the mitogenic effects associated with PAI-1.

Table of Contents

Title Page.....	I
Declaration.....	II
List of Publications.....	III
List of Conference Presentations.....	IV
List of Tables.....	V
List of Figures.....	VI
List of Abbreviations.....	IX
Acknowledgements.....	XII
Abstract.....	XIII
Table of Contents.....	XV
 1. Review of the Literature.....	 1
1.1 Introduction.....	2
1.2 The Plasminogen Activation System	3
1.2.1 Proteolytic Functions	3
1.2.2 Plasminogen/Plasmin	4
1.2.3 Urokinase Plasminogen Activator (uPA).....	7
1.2.4 Urokinase Plasminogen Activator Receptor (uPAR).....	11
1.2.5 Plasminogen Activator Inhibitors (PAI's)	16
1.2.6 Plasminogen Activator Inhibitor type 1 (PAI-1).....	19
1.2.7 Plasminogen Activator Inhibitor type 2 (PAI-2).....	22
1.2.8 PAI-2 and Cancer.....	29
1.3 Internalisation of plasminogen activation system components and Functional Consequences.....	33
1.3.1 The LDLR Family of Endocytosis Receptors.....	34
1.3.2 Caveolae Mediated Endocytosis	37
1.3.3 Receptor Mediated Endocytosis of uPA:PAI-1	41
1.3.4 Cell Signaling Through uPAR and the LDLR Family.....	44
1.4 Rationale and Aims of the thesis.....	47

2.	Characterisation of the Pathways of PAI-2 Internalisation	50
2.1	Introduction	51
2.2	Materials and Methods	52
2.2.1	Proteins, Antibodies and Reagents	52
2.2.2	Detection of Cell Surface Antigens by Dual Colour Flow Cytometry	53
2.2.3	Fluorescence Microscopy	54
2.2.2	Internalisation assays	56
2.2.3	BIACore Analysis of the Annexin II/PAI-2 Interaction	58
2.3	Results	59
2.3.1	Characterisation of the PC-3 Cell Line	59
2.3.2	Relaxed PAI-2 at the Cell Surface	61
2.3.3	Intracellular Localisation of Internalised PAI-2	61
2.3.4	Optimisation of Internalisation Assay	64
2.3.5	Visualisation and Quantification of PAI-2 Internalisation	66
2.3.6	Inhibition of PAI-2 Internalisation	69
2.3.7	Annexin II Dependent PAI-2 Internalisation	75
2.4	Discussion	77
3.	The Role of LRP in the Receptor Mediated Endocytosis of uPA:PAI-2	83
3.1	Introduction	84
3.2	Materials and Methods	86
3.2.1	Proteins, Antibodies and Reagents	86
3.2.2	Fluorescence Quenching Internalisation Assay	86
3.2.3	Co-localisation Studies using Confocal Microscopy	87
3.2.4	Binding of uPA:PAI-2 to Immobilised LRP	87
3.2.5	Surface Plasmon Resonance Analysis	88

3.3	Results	90
3.3.1	Candidate Endocytosis Receptors Involved in the Internalisation of PAI-2	90
3.3.2	The Interaction Between uPA:PAI-2 and LRP	93
3.3.3	Effect of uPAR on uPA:PAI-2 Binding to LRP.....	101
3.4	Discussion	101
4.	Structural basis of the differential signaling by PAI-1 and PAI-2 in Breast Cancer: Implications for metastatic potential	107
4.1	Introduction.....	108
4.2	Materials and Methods.....	110
4.2.1	Proteins and Antibodies	110
4.2.2	Tissue Culture Conditions.....	111
4.2.3	Analysis Of Cell Surface Antigen Expression And Internalisation By Flow Cytometry	111
4.2.4	Surface Plasmon Resonance Analysis	112
4.2.5	Plasmin Activity Assay	112
4.2.6	Confocal Microscopy Analysis of Cellular Phospho-Tyrosine Proteins	113
4.2.7	Analysis of ERK Activation	113
4.2.8	Cell Proliferation Assay	114
4.2.9	Protein Structure Analysis.....	115
4.3	Results	115
4.3.1	uPA:PAI-2 Endocytosis is Mediated by uPAR and VLDLr.....	115
4.3.2	PAI-2 Does Not Contain a High Affinity Binding Site for VLDLr.....	116
4.3.3	Structural Analysis of Serpin/VLDLr Binding	118

4.3.4	Serpin Internalisation is Related to VLDLr Affinity	124
4.3.5	PAI-2 Does Not Induce Mitogenic Signaling in MCF-7 cells.....	126
4.4	Discussion	131
5.	Conclusions and Future Directions	138
	REFERENCES	145
	APPENDIX 1: Buffers and Solutions	170
	APPENDIX 2: Dual colour flow cytometry	175
	APPENDIX 3: Fluorescence Quenching Internalisation Assay	176
	APPENDIX 4: Immobilisation on CM5 Sensor Chips	177
	APPENDIX 5: BIAcore Kinetic Analysis	178
	APPENDIX 6: Publications	180